Kawasaki Med. J. Vol. 2: No. 2, 81-89, 1976

RADIOIMMUNOASSAY FOR STEROID HORMONES I. RADIOIMMUNOASSAY FOR PLASMA CORTISOL

Seikoh NISHIDA, Shigeichi MATSUMURA, Masaharu HORINO, Hideki OYAMA and Atsuko TENKU
With the technical assistance of Kazuko OKAZAKI

Division of Endocrinology,
Department of Medicine, Kawasaki Medical School,
Kurashiki, Okayama, 701-01, Japan

Accepted for Publication on January 27, 1976

Abstract

A radioimmunoassay for the measurement of plasma cortisol has been developed. Antiserum raised against cortisol-21-hemisuccinate conjugated to BSA has a high specificity for cortisol. A useful range in a dose response line ranges from 10 pg to 500 pg. 0.002 ml of plasma is used for the measurement without chromatography. The coeffecient of variation for within assay and between assay is 8.4 % and 7.0 % in average. The method is sensitive and precise for practical use.

INTRODUCTION

For years, corticosteroids have been estimated in plasma as Proter-Silber chromogens or by their induced fluorescence. In 1963, Murphy et al.[1] first described the assay of cortisol using competitive protein binding assay (CPBA) and it is now widely used for the determination of plasma cortisol[1,2]. It offered an easier method for the assay of plasma cortisol, though it still has a few problems to be dissolved as for the specificity of corticosteroid binding globulin. Since then, in 1969, redioimmunoassay for estradiol-17β was reported by Anraham[3], radioimmunoassay techniques for steroid hormones were remarkably improved. A sensitive and precise radioimmunoassay for plasma cortisol by using highly specific antiserum for cortisol prepared in rabbits will be described.

MATERIALS AND METHODS

1. Chemicals. Cortisol-1, 2, 6, 7-^{3}H, 82.7 Ci/mM (New England Nuclear Corp.) was used after purification with thin-layer chromatography. Authentic cortisol was purchased from Sigma. A series of steroids checked for cross-reaction were kindly donated by Teikoku Zoki Pharmaceutical Co., Japan. Bovine serum albumin fraction V (Sigma, BSA),

bovine gamma globulin fraction II (Miles Laboratories, inc.) and Freund's complete adjuvant (Difco) were purchased at market. All other chemicals were of reagent grade.

2. Antigen. Cortisol (600 mg) and succinic anhydride (600 mg) were dissolved in 8 ml pyridine, kept standing overnight, precipitated with 4N-HCl after addition of 10 ml of cooled water. About 500 mg of cortisol-21-hemisuccinate was obtained after recrystallization with acetone. Conjugation of cortisol-21-hemisuccinate to BSA was performed according to the method of Erlanger et al[4,5].

3. Antiserum. Rabbits were immunized every four weeks by multiple subcutaneous injections of 1 mg of cortisol-21-BSA dissolved in saline, emulusified in Freund's complete adjuvant. Antibodies to cortisol were detectable after 3 months of immunization. Antisera were collected 10 days after the last immunization.

4. Sample preparation. 0.1 ml of plasma was added to 9.9 ml of water and mixed. 0.2 ml aliquot of the diluted plasma (0.002 ml of plasma) was transferred to the assay tube containing dried 1,000 dpm of ^{3}H-

TABLE 1

Radioimmunoassay Procedure for Plasma Cortisol

diluted plasma 0.2 ml (plasma 0.002 ml) and
ether 1 ml, with dried 1,000 dpm of ^{3}H-cortisol
↓ extract, 30 seconds by vortex mixer
after dryness, divide the cortisol into two parts
with 0.4 ml of methanol

Recovery	Assay
counting vial	^{3}H-cortisol, 10,000 dpm
containing	↓ evaporate in N_2 gas
10 ml of scintillator	antiserum (1:55,000), 0.25 ml
	↓ mix, 15 seconds,
	↓ incubate, 30 min.
	↓ at room temperature
	saturated $(NH_4)_2SO_4$, 0.2 ml
	↓ stand still, 10 min.
	↓ at room temperature
	↓ centrifuge, 10 min., 3,000 rpm,
	0.2 ml of supernatant into counting vial
	containing 10 ml of scintillator

cortisol. The cortisol was extracted into 1 ml of ether and washed by 0.2 ml of water. After aspiration of the water layer to dryness, the cortisol was divided into two parts, one for assay, the other for recovery counts into counting vial.

5. Assay procedure. The assay procedure was shown in Table 1. The assay was performed by the modified Makino and Kambegawa's method[6].

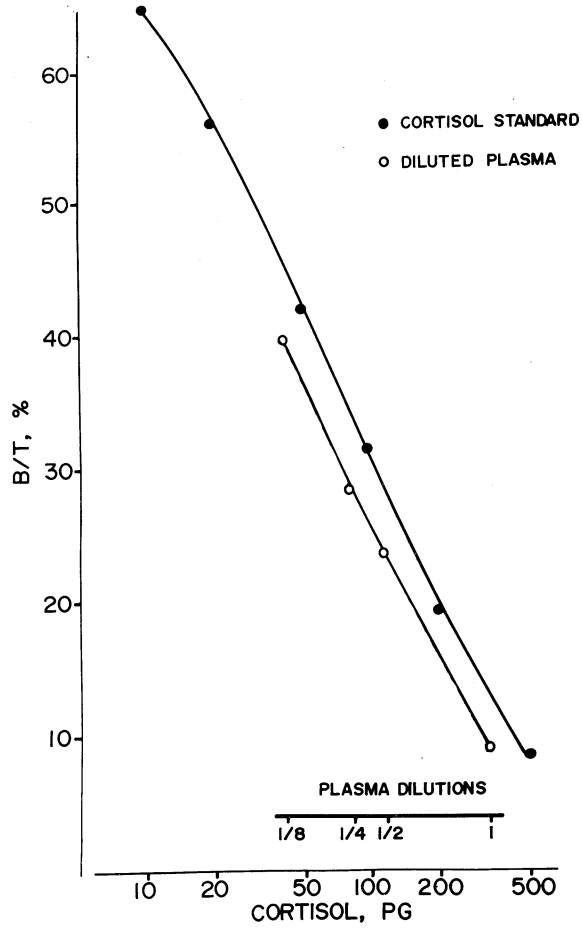

Fig. 1. A typical dose response line and dilution test on semi-logarithmic scale. Dose response line; each black circle represents the mean from duplicate determinations. The final dilution of anti-cortisol-3 is 1:55,000. Dilution test; original plasma obtained from a patient with Cushing's syndrome, of which concentration of cortisol was 335 ng/ml, was diluted to 1:2, I:4, 1:8. Each open circle represents the mean from four determinations.

The entire assay procedure was carried out in 8 mm×85 mm glass tubes. ^{3}H-cortisol was diluted with methanol, original anti-cortisol serum was diluted to 1 : 50,000 with 0.05 M borate buffer (P^{H}8.0) containing 0.05 % BSA and 0.075 % bovine gamma globulin. For standard curve, diluted cortisol was added at the concentrations of 10, 20, 50, 100, 200 and 500 pg.

6. Radiocount. Radioactivity was measured with 10 ml liquid scintillation solution, 10 g PPO, 250 mg POPOP, 100 g Naphthalene in 1 liter dioxane. Each sample was counted in a Model 3385 Packard Tri-Carb liquid scintillation spectrometer. The mean efficiency was 52 % for ^{3}H.

7. Calculation. From A (^{3}H dpm used in assay) and P (^{3}H dpm of sample), bound per cent (B) was obtained by following formula, B (%)= (A-P×0.45/0.2)×100/A. From the formula, cortisol level per 0.002 ml plasma (M) was read from standard curve. Finally, cortisol level (ng/ml) was obtained from following formula, cortisol (ng/ml)=M×100/recovery %×2×10^{3}/0.002.

8. 11-OHCS determination. In a serial group, plasma 11-hydroxycorticosteroid was determined by De Moor's method[7].

RESULTS

1. A typical dose response line and sensitivity. A typical dose response line is shown in Fig. 1. Bound per cent for total ^{3}H-cortisol was 67.5 % in duplicate at 0 pg of standard cortisol and was 8.5 % at 500 pg of added cortisol. Practical sensitivity is 10 pg in the assay.

2. Dilution test. The results of dilution test of the assay is shown in Fig. 1. Original plasma, of which concentration of cortisol was 335 ng/ml, was diluted to 1 : 2, 1 : 4, 1 : 8, and these diluted plasma showed the parallel curve with authentic standard curve.

3. Precision and reprodubility. Precision (within assay) and reproducibility of the assay are summarized in Table 2. Average precision as defined by the coefficient of variation was 8.4 % among four different plasma samples. The average coefficient of variation on between assay done in 3 different occasions was 7.0 % for five different plasmas.

4. Recovery. In two different plasmas, average total recoveries were 98.8 % and 101.3 % (Table 3).

5. Specificity. The cross-reactivity of the anti-cortisol serum with various steroids are shown in Table 4. 11-deoxycortisol and 17-OH-progesterone crossreacted 23.6 % and 22.4 %, respectively. Also, cortisone and corticosterone showed cross-reactivity above 10 %. However,

TABLE 2

Precision and Reproducibility of Plasma Cortisol Radioimmunoassay

Smple	Cortisol, ng/ml (Average)	CV, %
Within assay (N=6)		
A	25	5.7
B	68	11.0
C	156	7.1
D	245	9.8
		8.4 (average)
Between assay (3 different occasions)		
E	30	19.8
F	66	1.5
G	77	4.9
H	114	5.4
I	309	3.6
		7.0 (average)

TABLE 3

The Accuracy of Recovery of Added Cortisol from Plasma

Plasma sample	Cortisol added (pg)	Cortisol determined (pg)	Recovery (%)	Plasma sample	Cortisol added (pg)	Cortisol determined (pg)	Recovery (%)
I	0	66		II	0	414	
	20	86	100.0		20	476	109.7
	50	119	102.5		50	472	101.7
	100	142	85.5		100	490	95.3
	200	289	108.9		200	592	96.4
	500	550	97.2		500	945	103.3
			98.8±7.7 (M±SD)				101.3±5.2 (M±SD)

Plasma sample; diluted plasma 0.2 ml (plasma 0.002 ml).
Cortisol determined; mean from four determinations.

they are extremely low in peripheral circulation compared with cortisol level. Thus, chromatographic purification of cortisol can be eliminated except for special example like adrenogenital syndrome.

6. As well known, 11-OHCS determination by fluorimetry measures cortisol, corticosterone and other metabolites[7], therefore, the determina-

TABLE 4

The Cross Reactivity of Anti-Cortisol-3 (1 : 55,000)

Steroids	Cross-reactivity, %
11-DEOXYCORTISOL	23.6
17-OH-PROGESTERONE	22.4
CORTISONE (E)	18.2
CORTICOSTERONE (B)	14.2
PROGESTERONE	5.8
DOC	5.7
ALDOSTERONE	5.2
TESTOSTERONE	4.3
ANDROSTENEDIONE	3.0
DEHYDROCORTICOSTERONE (A)	2.9
PREGNENOLONE	1.8
DHEA	< 1.0
ESTRONE (E_1)	〃
ESTRADIOL (E_2)	〃
ESTRIOL (E_3)	〃
17-OH-PREGNENOLONE	〃
PREGNANETRIOL	〃
ETIOCHOLANOLONE	〃
5α-PREGNANEDIONE	〃
5β-PREGNANEDIOL	〃
ANDROSTERONE	〃
DIHYDROTESTOSTERONE	〃
EPIESTRIOL	〃
TETRHYDROCORTISOL (THF)	〃
DEXAMETHASONE	1.5

TABLE 5

Rapid ACTH Test in 6 Normal Subjects, Comparison of Cortisol Values Obtained by Radioimmunoassay and 11-OHCS Determination

Case	Before	Cortisol / 11-OHCS	30 min. after ACTH	Cortisol / 11-OHCS	60 min. after ACTH	Cortisol / 11-OHCS
1.	17.0(20.3)	83.7	25.8(30.5)	84.6	29.6(33.9)	87.3
2.	17.1(21.2)	80.7	36.3(44.0)	82.5	43.8(55.0)	79.6
3.	19.5(25.2)	77.4	32.8(45.0)	72.9	38.4(50.4)	76.2
4.	11.5(14.0)	82.1	28.2(32.1)	87.9	33.7(37.1)	90.8
5.	12.2(19.0)	64.2	21.8(31.0)	70.3	26.7(37.0)	72.2
6.	10.6(15.0)	70.7	31.0(35.9)	86.4	33.0(44.1)	74.8
		76.5±6.9 (M±DS)		80.8±6.7 (M±SD)		80.2±6.7 (M±SD)

Cortisol, μg/100 ml; ( ) 11-OHCS, μg/100 ml; $\frac{\text{Cortisol}}{\text{11-OHCS}}$, %

Plasma samples were collected before, 30 min., and 60 min. after intramuscular injection of 0.25 mg Cortrosyn.

tion is not so specific for cortisol measurements as radioimmunoassay. In Table 5, simultaneously determined cortisol and 11-OHCS values are listed. In 6 normal subjects, cortisol/11-OHCS was 76.5 % before ACTH injection, 80.0 % 30 min. after, 80.2 % 60 min. after ACTH. There was no significant difference among them. Thus, cortisol occupies about 80 % of plasma 11-OHCS values.

7. The results of cortisol assay in normal subjects, Cushing's syndrome, simple obesity and Sheehan's syndrome are shown in Fig. 2. In normal subjects cortisol value was 142.4±37.2 ng/ml (M±SD), ranging from 83 to 228 ng/ml at 9 A. M. and 133.8±32.8 ng/ml in four simple obesity, whereas a Cushing's syndrome showed 505 ng/ml, and a Sheehan's syndrome 13 ng/ml.

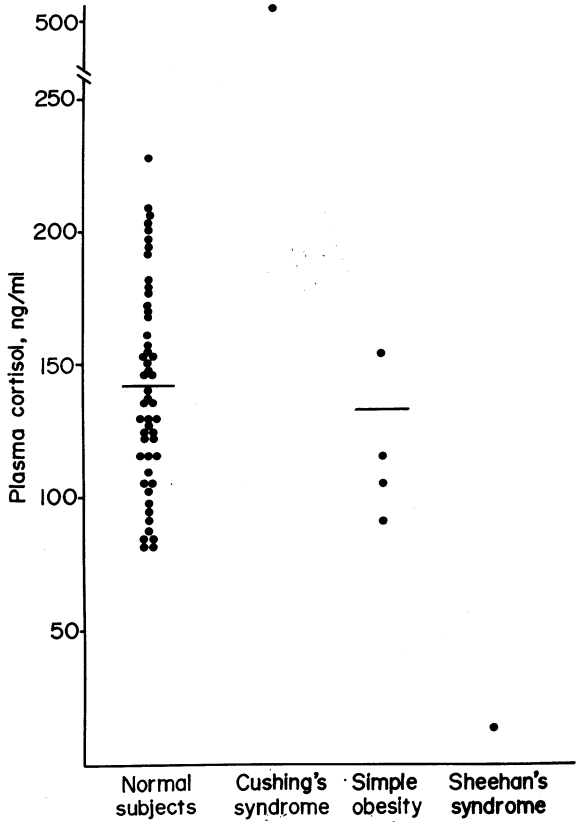

Fig. 2. The cortisol values of the assays in 50 normal subjects, Cushing's syndrome, 4 cases of simple obesity, and Sheehan's syndrome. Each bar represents the mean value.

DISCUSSION

Many methods have been described for the determination of plasma cortisol. The two in current use are the 11-OHCS determination by fluorimetry and competitive protein binding assay. The fluorimetry for plasma cortisol is easy to perform but is not specific unless prior chromatography is undertaken[8]. Competitive protein binding assay has been successfully applied to measurement of plasma cortisol[1,2]. For the following reasons, Ruder et al.[8] pointed out the radioimmunoassay's superiority to the competitive binding assay.[1] The binding affinity of corticosteroid binding globulin (CBG) is only 1/100 that of cortisol antiserum. Because of this, the practical sensitivity of CBG-assay is limited to 1.0 ng.[2]. Therefore, CBG-assay has a standard curve with a small useful range, usually from 2 to 8 ng[3]. Chromatographic systems often give high blank values[4]. Separation of bound and free has been a major problem with the CBG-assay. Ammonium sulfate or charcoal separation of bound and free hormones for radioimmunoassay is rapid and convenient. In the present study, cortisol occupied about 80 % of 11-OHCS values, furthermore, in Makino et al.'s report[6] radioimmunoassayed cortisol stood for only 50 % of fluorimetried cortisol. And, Ruder et al.[8] compared cortisol values obtained by RIA and by CBG-assay. Average value of 27 samples obtained by CPBA was 18.9 μg/100 ml; average by RIA was 16.7 μg/100 ml. As for the antigen for cortisol antiserum, Ruder et al.[8], Makino et al.[6] also used cortisol-21-hemisuccinate-BSA as immunizing antigen. The antiserum of Ruder et al.[8] cross-reacted 100 % with 11-deoxycortisol and 21-deoxycortisol, and more than 40 % with 17-OH-progesterone, corticosterone, DOC and cortisone. Utilizing the above mentioned antiserum, Ruder et al. compared the values obtained by with-, and without-chromatography step, and reported that mean difference was 8 ng in 30 normal subjects[8]. And they conclude that only in patients, such as congenital adrenal hyperplasia, adrenal carcinoma, or in normal subjects treated with metyrapone, chromatographic step will be necessary. Although our more specific antibody can eliminate chromatographic purification of cortisol from small amounts of peripheral, circulating 11-deoxycortisol or 17-OH-progesterone, in above mentioned patients the concentrations of cross-reacting steroids would be high enough to necessiate a chromatography. The plasma cortisol values obtained in the present study agree with those previously reported[8,9].

Acknowledgment

The authors thank Dr. Makino and Dr. Kambegawa for their gifts of various authentic steroids and their valuable suggestions. This investigation was supported in part by the Research Project Grant of the Kawasaki Medical School (50-505).

REFERENCES

1) Murphy, B. E., Engelberg, W. and Pattee, C. J.: J. clin. Endocr. 23: 293-300, 1953.
2) Murphy, B. E., Engelberg, W. and Pattee, C. J.: J. clin. Endocr. 27: 973-990, 1967.
3) Abraham, G. E.: J. clin. Endocr. 29: 866-870, 1969.
4) Erlanger, B. F., Borek, F., Beiser, S. M. and Lieberman, S.: J. Biol. Chem. 228: 713-727, 1957.
5) Erlanger, B. F., Borek, F. and Lieberman, S.: J. Biol. Chem. 234: 1090-1094, 1958.
6) Makino, T. and Kambegawa, A.: Folia Endocr. Japonica. 49: 1297-1306, 1973 (in Japanese)
7) De Moor, P., Steeno, O., Raskin, M. and Hendrikx, A.: Acta endocr. 33: 297-307, 1960.
8) Ruder, H. J., Guy, R. L. and Lipsett, M. B.: J. clin. Endocr. 35: 219-224, 1972.
9) Demura, H.: Folia Endocr. Japonica. 49: 127-128, 1973 (in Japanese).